

Wound healing in Mac-1 deficient mice

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ABSTRACT

Mac-1 (CD11b/CD18) is a macrophage receptor that plays several critical roles in macrophage recruitment and activation. Because macrophages are essential for proper wound healing, the impact of Mac-1 deficiency on wound healing is of significant interest. Prior studies have suggested that Mac-1^{-/-} mice exhibit deficits in healing, including delayed wound closure in head and ear wounds. The current study examined whether Mac-1 deficiency influences wound healing in small excisional and incisional skin wounds. Three millimeter diameter full thickness excisional wounds and incisional wounds were prepared on the dorsal skin of Mac-1 deficient (Mac-1^{-/-}) and wild-type (WT) mice, and wound healing outcomes were examined. Mac-1 deficient mice exhibited a normal rate of wound closure, generally normal levels of total collagen, and nearly normal synthesis and distribution of collagens I and III. In incisional wounds, wound breaking strength was similar for Mac-1^{-/-} and WT mice. Wounds of Mac-1 deficient mice displayed normal total macrophage content, although macrophage phenotype markers were skewed as compared to WT. Interestingly, amounts of TGF- β 1 and its downstream signaling molecules, SMAD2 and SMAD3, were significantly decreased in the wounds of Mac-1 deficient mice compared to WT. The results suggest that Mac-1 deficiency has little impact on the healing of small excisional and incisional wounds. Moreover, the findings demonstrate that the effect of single genetic deficiencies on wound healing can markedly depend upon the wound model. These conclusions have implications for the interpretation of the many prior studies that utilize a single model system to examine wound healing outcomes in genetically deficient mice.

INTRODUCTION

Wound healing is a complex yet well-regulated process in which multiple resident cells, recruited inflammatory cells, and stem cells interact to create an environment that supports the healing process. An optimal inflammatory response is a normal and important part of the healing process that helps to eliminate contaminating microorganisms and dead or injured cells.^{1, 2}

Multiple prior studies have demonstrated that appropriate inflammation is critical to healing outcomes, and several specific immune cell types have been shown to play critical roles.

Mac-1 ($\alpha_M\beta_2$ integrin, CD11b/CD18, or CR3) is a β_2 integrin cell-surface receptor composed of α_M - and β_2 -subunits. Mac-1 is primarily found on myeloid cells including monocytes, macrophages, neutrophils, and some lymphocytes. It binds to a variety of soluble matrix-bound or cell-bound ligands, such as complement iC3b, fibrinogen, factor X, heparin/heparin sulfate, and intercellular adhesion molecule 1. Via these ligands, Mac-1 mediates multiple leukocyte functions, including activation, adhesion, diapedesis, cell motility, cell-to-cell interaction, and phagocytosis.³⁻⁵

Two specific Mac-1 positive cell types, neutrophils and macrophages, are known to be important to wound healing. Neutrophils are among the first leukocytes to infiltrate the wound, entering the site within minutes to hours after injury.¹ Neutrophils are known to be important in eliminating microbes and engulfing dead or damaged cells in the wound. However, neutrophils may also damage normal tissue through the release of reactive oxygen species and proteases.⁶ Neutrophil depletion has been shown to accelerate wound reepithelialization in mice, yet to have

little effect on collagen deposition, wound breaking strength and macrophage infiltration.⁷ In contrast to neutrophils, which function primarily at early time points after injury, macrophages play a pivotal role in all stages of skin wound healing.^{8,9} Wound macrophages exhibit extraordinary phenotypic plasticity that is essential to their function.¹⁰⁻¹³ In the early stage of wound healing, macrophages are in a primarily pro-inflammatory state (often termed M1 or classically activated macrophages), producing IL-1 β , IL-6, and TNF- α .^{11,12} As the wound heals and enters the stages of cellular proliferation and remodeling, macrophages adopt a reparative phenotype (often termed M2 or alternatively activated macrophages), producing factors that support tissue growth and reduce inflammation.^{11,12} Several studies have demonstrated that macrophage function is essential for healing, and some have suggested that the reparative phenotype macrophage is particularly critical for adequate repair.¹⁰⁻¹² More recent studies have established that macrophages are in fact a heterogeneous cell population at all stages of the wound healing process,¹⁰⁻¹² raising questions about what phenotypes are most beneficial. Although several prior studies suggest that the addition of activated macrophages can improve healing outcomes,¹⁴ other studies have demonstrated that the treatment of wounds with M2 macrophages does not benefit wound healing.¹⁵

Given the importance of neutrophils and macrophages to the healing process, and the critical role that Mac-1 plays in the activation, adhesion, and migration of these cells, Mac-1 might be expected to play an important role in wound repair. Indeed, two prior studies, one using ear and head wounds, and the other examining 5mm excisional skin wounds, have demonstrated that specific strains of Mac-1 deficient mice exhibit delayed wound healing.^{16,17} In the current study, we used a commercially available Mac-1 deficient strain to examine whether this deficit

extends to slightly smaller wounds and incisional wounds produced on the dorsal skin. The current results show that although small excisional wounds of Mac-1 mice exhibit some functional changes related to macrophages and decreased levels of TGF- β 1, wounds on these mice heal nearly normally. The results also demonstrate that incisional wounds heal with normal breaking strength in Mac-1 deficient mice. The data has important implications for the interpretation of wound healing outcomes in genetically deficient mouse strains.

MATERIALS AND METHODS

Animals

Nine to ten-week old female Mac-1 $-/-$ (CD11b null) mice on a C57BL/6 background and WT C57BL/6 were used in the current study. All mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). The absence of CD11b/Mac-1 in Mac-1 $-/-$ mice was confirmed by immunohistochemistry of sections from day 1 wounds of WT and Mac-1 $-/-$ mice with CD68 (macrophages) or Gr-1 (neutrophils) and CD11b antibodies as described below (see Indirect immunofluorescence). CD11b was absent on both neutrophils and macrophages in Mac-1 $-/-$ mice (Fig.1 A and B).

Wound models

Six 3mm full thickness excisional wounds were made on dorsal skin using a standard biopsy punch under ketamine (100mg/Kg) and xylazine (5mg/Kg) anesthesia. The wounds of 10 mice from each group were photographed at time zero and then every day post wounding until the wounds healed. Wound sizes were determined by software AxioVision (Zeiss, Oberkochen, Germany). The percent of wound closure was calculated as previously described.^{18, 19} Out of six

wounds on each mouse, three wounds were frozen and stored at -80°C for ELISA, one wound was stored in RNAlater (Sigma Aldrich, St. Louis, MO) for total RNA extraction and real-time PCR, 1 wound was embedded in OCT compound and frozen for immunostaining, and one wound was fixed in formalin for histologic analysis. To evaluate wound breaking strength, a single 2cm incisional wound was made on the dorsal skin of Mac-1 ^{-/-} and WT mice while under anesthesia. Incisional wounds were closed using two surgical clips that were removed at day 5 post-wounding. All studies were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee and the U.S. Army Animal Care and Use Review Office.

Wound breaking strength

Skin strips that spanned the 2cm incisional wounds were prepared at days 7, 14, 21, and 28 post-wounding. Breaking strength (in lb) was recorded as weight load at the point of wound breakage using a motorized tensiometer (Mark-10, Copiague, NY) as described previously.^{19, 20} Two wound strips per mouse were subjected to analysis and the average was recorded as the wound breaking strength for that individual animal. Normal skin from unwounded mice was also tested. N (number of mice) = 6 at each time point and in each group.

Indirect immunofluorescence

For immunofluorescent staining of macrophages, neutrophils, and myofibroblasts, 8µm frozen tissue sections were air-dried, fixed in cold acetone for 10 min, and blocked with 10% goat serum for 30 min. Sections were then incubated with rat anti-mouse CD68 (Abcam, Cambridge, MA), rat anti-mouse Gr-1 (BD Biosciences, San Jose, CA), rabbit anti-mouse α -smooth muscle actin (α -SMA) (for myofibroblast staining, Abcam, Cambridge MA) or rabbit anti-mouse

CD11b (Abcam, Cambridge, MA) for 45 min followed by incubation with Alexa fluor 594 goat anti-rat IgG, Alexa fluor goat anti-rat or rabbit IgG 488 (Invitrogen, Carlsbad, CA), respectively. The staining procedures were all performed at room temperature. Stained sections were evaluated using a fluorescence microscope, Axioskop 40 (Zeiss, Oberkochen, Germany) and recorded with a digital camera, AxioCam MRc (Zeiss, Oberkochen, Germany). Gr-1 positive cells in the wounds and wound margins were counted and the average number per 20x field was calculated. The density (% positive staining in wound margin and wound bed) of CD68 and α -SMA was quantified using Image J.²¹ N (number of mice) = 6 at each time point and in each group.

Hematoxylin/eosin (HE) staining

Five μ m thick paraffin-embedded skin tissues were stained with HE using a standard protocol.¹⁹ The sections were examined with an Axioskop 40 microscope (Zeiss, Oberkochen, Germany). Images were captured with 5x objective lenses. The rate of re-epithelization based on HE stained wound sections was calculated according to our previously described method.^{18, 19} For re-epithelialization measurements, N (number of mice) = 6 at each time point and in each group except for n=5 for WT at day 3. The volume of granulation tissue in wound bed between wound edges on days 5 and 7 post wounding was also measured using AxioVision software (Zeiss, Oberkochen, Germany). For the measurement of granulation tissue, N (number of mice) = 6 at each time point and in each group except for n=5 for Mac-1^{-/-} at day 5.

Real time PCR

Total RNA was extracted from normal skin (unwounded skin) and wounds of Mac-1 ^{-/-} and WT mice using TriZol (Invitrogen, Carlsbad CA). One µg of each sample was treated with DNase I (Invitrogen, Carlsbad CA) to remove any contaminating DNA, and then subjected to reverse transcription using a Retro-script kit (Invitrogen, Carlsbad CA). Relative mRNA expression of collagen I, collagen III, YM1, iNOS, SMAD2, and SMAD3 was examined using a SYBR green PCR mix (Roche, Basel, Switzerland) and gene specific primers by a real-time PCR system (StepOne Plus, Applied Biosystems, Carlsbad, CA). GAPDH was used as a housekeeping gene for calibration. Primer sequences are listed in Table 1. N (number of mice) = 6 at each time point and in each group.

ELISA and Multiplex ELISA

Skin wounds tissue from WT and Mac-1 ^{-/-} mice was homogenized in ice cold PBS with a protease inhibitor cocktail for mammalian cells (Sigma Aldrich, St. Louis MO) followed by sonication. The samples were centrifuged at 16,000xg for 15 minutes. The supernatants were collected and stored at -80°C until use. Protein expression of CCL-2, CCL-3, CXCL1, IL-1β, IL-6, and TNF-α was determined using a multiplex ELISA kit (eBioscience, San Diego, CA). TGF-β1 protein expression was also examined by an ELISA kit (eBioscience, San Diego, CA). Assays were performed according to the manufacturers' instructions. N (number of mice) = 6 at each time point and in each group.

Hydroxyproline analysis

Wounds from WT and Mac-1 ^{-/-} mice were excised on days 7, 14, 21 and 28 after injury, weighed, and stored at -80°C until analysis. Both wounded and unwounded skin tissues were hydrolyzed in 1 ml of 6 N HCl overnight at 95°C for 20 hours. The hydroxyproline content was then analyzed using a hydroxyproline assay kit (QuickZyme Biosciences, Leiden, Netherlands). Results are expressed as nM/mg tissue.

Collagen analyses with picrosirius red and Masson's trichrome

Five µm thick paraffin-embedded skin tissues were stained with Masson's trichrome and picrosirius red using standard protocols.¹⁹ The relative density of blue-stained collagen under Masson's trichrome staining was quantified in the wound bed at days 7, 14, 21, and 28 using Image J. Mature collagen (collagen I, red) and immature collagen (collagen III, green) were quantified using Image J analysis of picrosirius red stained sections. The percent of mature or immature collagen was calculated as follows: pixels of mature or immature collagen/total pixels of mature and immature collagen x100. N (number of mice) = 5 at each time point and in each group.

Statistical analyses

Data are expressed as mean \pm SEM. Two-way ANOVA followed by Bonferroni's multiple comparisons test or t test was used for statistical analysis using software GraphPad Prism (GraphPad Software, Inc. La Jolla, CA). P values less than 0.05 were considered statistically significant.

RESULTS

Mac-1 deficiency does not affect wound closure but reduces granulation tissue formation.

To compare wound healing in small excisional wounds in Mac-1 $-/-$ and WT mice, wound size was measured every day after wounding until fully closed. Wounds in Mac-1 deficient mice closed at the same rate as WT mice (Fig. 1C&E). Histological analysis confirmed that there was no significant change in wound re-epithelization between these two groups of mice ($p>0.05$, Fig. 1D&F). When granulation tissue was measured, the volume of granulation tissue in the wound bed of Mac-1 deficient mice was about half that of WT mice at day 7 ($p<0.05$, Fig. 1G).

Changes in macrophage phenotype but not quantity occur in the wounds of Mac-1 deficient mice.

We next examined possible changes in macrophage infiltration and function in excisional skin wounds of Mac-1 deficient mice. In wounds of both WT and Mac-1 $-/-$ mice, the number of macrophages markedly increased from days 3 to 7 post-wounding and then slowly returned to baseline (Fig. 2A). No significant difference in macrophage content was observed between the two groups at any time point. This observation was paralleled by the minimal changes in the level of the macrophage chemoattractant CCL-2 when compared between the two strains. A single significant difference was found; CCL-2 was markedly decreased in Mac-1 $-/-$ mice at day 1 post-wounding ($p<0.001$), a time point well in advance of peak macrophage content (Fig. 2B).

Next we examined whether the generation of macrophage phenotypes might be altered in wounds of Mac-1 $-/-$ mice. To assess and compare the macrophage phenotypic assortment in

wounds, we first measured the expression of a frequently used M2 marker, YM1. As compared to WT mice, YM1 expression was significantly decreased at days 1, 3, and 7 post injury in the wounds of Mac-1 ^{-/-} mice, reaching statistical significance at days 1 and 7 (p<0.001) (Fig. 2C). Interestingly, the pro-inflammatory (i.e., M1) macrophage marker iNOS was also significantly downregulated on days 3, 7, and 14 post-wounding in wounds of Mac-1 ^{-/-} mice compared to WT mice (Fig. 2D, p<0.001). Taken together, these data suggest that when compared to WT, macrophage phenotypes and the phenotypic transition may be skewed in the wounds of Mac-1 ^{-/-} mice.

Neutrophil content and proinflammatory mediators in wounds of Mac-1 ^{-/-} mice

The number of neutrophils in the wounds of Mac-1 ^{-/-} and WT mice were compared by quantifying Gr-1 positive cells in the wound bed. The number of neutrophils was slightly but significantly greater in the wounds of Mac-1 ^{-/-} mice than those of WT mice at the single time point of 12-hours (Fig. 3A, p<0.05), but this difference quickly resolved. Wounds of WT and Mac-1 ^{-/-} mice showed no differences in the amount of the key neutrophil chemoattractant CXCL-1 (Fig. 3B).

The amounts of three other well-described inflammatory cytokines, IL-1 β , IL-6, and TNF- α , were also compared in wounds from the two strains. Each of these cytokines showed an expected increase and decrease as wound healing progressed in both strains. Only a single significant difference was seen between Mac-1 ^{-/-} and WT over the time course of wound healing for these three cytokines (Fig. 3, C, D & E). IL-6 was significantly lower in wounds of Mac-1 ^{-/-} mice than in WT mice at day 1 post-wounding (p<0.01) (Fig. 3D).

Mac-1 deficient mice exhibit minimal changes in wound collagen synthesis and architecture.

To determine if Mac-1 deficiency might affect collagen synthesis in wounds, we measured hydroxyproline as a marker of total collagen content. The concentration of hydroxyproline in the wounds was slightly lower in Mac-1 $-/-$ mice than that of WT mice at days 14, 21 and 28. However, this difference reached statistical significance only at day 14 ($p < 0.05$, Fig. 4A).

To further understand if the production of major collagens (i.e., collagen I and III) involved in the wound healing process were altered by Mac-1 deficiency, collagen mRNA expression was measured by quantitative RT-PCR. The expression of collagen I mRNA was low in the early wounds of both strains of mice, reaching a peak at day 7 in WT mice and day 14 in Mac-1 $-/-$ mice, respectively (Figure 4B). Collagen III expression was also low at early time points, peaking on day 7 in WT mice and on day 21 in Mac-1 $-/-$ mice, respectively (Fig. 4C). At the single time point of day 7, the expression of both collagens I and III was significantly higher in WT mice than in Mac-1 $-/-$ mice ($p < 0.01$ and 0.05 for collagen I and collagen III, respectively, Fig. 4B&C). This difference was not sustained, as the expression of both collagens I and III showed no statistically significant difference between Mac-1 $-/-$ and WT mice at days 14 and 21 ($p < 0.05$) (Fig. 4 B&C).

Histologic analysis of collagen showed no differences between WT and Mac-1 deficient mice.

To further assess whether any substantive differences in collagen production occurred in the wounds of Mac-1 ^{-/-} and WT mice, collagen content was evaluated histologically using Masson's trichrome and picrosirius red staining. Quantification of trichrome staining in wounds of Mac-1 ^{-/-} and WT mice showed no significant differences (Fig. 5A&B). Picrosirius red staining was also examined as this stain allows for the quantification of the relative amounts of collagen I and collagen III. The picrosirius staining revealed no statistically significant difference in the relative levels of Collagen I and Collagen III in wounds from the two strains of mice at any time point. Unwounded skin from both WT and Mac-1 ^{-/-} mice contained approximately 90% collagen I (red-orange) and 10% collagen III (green) (Fig. 5C&D). By day 7 after wound placement, collagen III was predominant in both strains. After day 14, the level of collagen I gradually increased as collagen III gradually decreased in both strains. By day 28, wounds of both strains contained about 80% collagen I and 20% collagen III. Therefore, the collagen in wounds from WT and Mac-1 ^{-/-} mice underwent the same temporal transition from primarily immature type III collagen to mature type I collagen fibers.

Myofibroblast levels and wound breaking strength are similar in Mac-1 deficient and WT mice.

Because myofibroblasts are involved in wound contraction, collagen synthesis, and scar formation, the density of these cells was compared in wounds of Mac-1 ^{-/-} and WT mice using α -SMA as a marker. When compared to WT, wounds of Mac 1 ^{-/-} mice exhibited a noticeable but insignificant decrease in α -SMA⁺ myofibroblasts than Mac-1 ^{-/-} mice on day 7 (p=0.3, Fig. 6A).

To further examine dermal repair, we tested wound breaking strength in Mac-1 ^{-/-} and WT mice using an incisional wound model. In keeping with the minimal changes in collagen content that were observed in excisional wounds (Fig. 4&5), no difference in incisional wound breaking strength was observed between the two mouse strains on days 7, 14, 21, or 28 post-wounding (Fig. 6B).

TGF-β1 production was significantly impaired in Mac-1 deficient mice

Given the critical role of TGF-β1 in wound repair, we next compared TGF-β1 production in the wounds of Mac-1 ^{-/-} and WT mice. TGF-β1 concentration was more than fourfold lower in wounds of Mac-1 ^{-/-} versus WT mice (day 7, 121.7±18.8 pg/mg vs. 25.9±10.7 pg/mg, p<0.001, Fig. 7A). Furthermore, mRNA analysis showed that the expression of SMAD2 and SMAD3, two molecules in the TGF-β1 signaling pathway, was also significantly lower in wounds of Mac-1 ^{-/-} mice (Fig. 7B).

DISCUSSION

In the current study, we sought to investigate the impact of Mac-1 deficiency on mouse skin wound healing using small excisional and incisional skin wound models. Rather surprisingly, the results of this study demonstrate that small excisional wounds on Mac-1 deficient mice exhibit no substantial impairment in wound closure or collagen synthesis. The few changes that were significantly different in the wounds of Mac-1 ^{-/-} mice were often seen only at single time points. For example, at limited and specific time points, amounts of collagen I and collagen III mRNA as well as total collagen content were significantly decreased in wounds of Mac-1 ^{-/-} mice compared to WT mice. One interesting difference was that the production of TGF-β1 was

significantly decreased in the wounds of Mac-1 $-/-$ mice; a similar decrease was seen in two TGF- β 1 downstream signaling pathway molecules, SMAD2 and SMAD3. TGF- β 1 contributes to the recruitment of fibroblasts and stimulates the synthesis of collagens I, III, proteoglycans, fibronectin, and other extracellular matrix components through the SMAD2 and 3 signaling pathway.^{22, 23} The observation of generally normal wound closure and collagen synthesis in the face of TGF- β 1 deficiency demonstrates that the Mac-1 $-/-$ mice were capable of compensating for this deficit in the wound models employed in this study.

One of the more striking observations of our study was a significant change in macrophage phenotype in the wounds of Mac-1 $-/-$ mice. Using the markers YM1 (for M2 like macrophages) and iNOS (for M1 like macrophages), wounds of Mac-1 $-/-$ mice were shown to have significantly lower levels of macrophages of both phenotypes. With the caveat that our studies examined only single markers, these results suggest that the wounds of Mac-1 $-/-$ mice are characterized by an unusual pattern of macrophage phenotypes. The concept that macrophages in the wounds of Mac-1 $-/-$ mice exist primarily in non-traditional phenotypes can be explained by emerging studies on the complexity of this cell type. Recent studies have demonstrated that macrophage phenotypes might be best considered in a multi-dimensional model.²⁴ In particular, the analysis of genome-wide transcriptional profiling of macrophages now suggests a very complex macrophage response to stress signals.²⁵ What is most interesting in the current study is that despite apparent changes in macrophage functionality, small excisional wounds healed quite normally in Mac-1 deficient mice. This result provides supporting evidence that our current understanding of importance of specific macrophage phenotypes in wound healing is incomplete.

Our study demonstrates that infiltration of macrophages into wounds, in terms of total numbers, was unaffected in Mac-1 $-/-$ mice compared to wild type mice. Thus macrophage recruitment to wounds, and perhaps emigration from the wound site, appear to be independent of Mac-1 for skin wounds. This finding is similar to prior wound healing studies in Mac-1 deficient strains. Those previous investigations also reported minimal changes in macrophage content in skin injury sites.^{16, 17} In contrast, in an induced peritoneal inflammation model, Mac-1 $-/-$ mice exhibited enhanced macrophage accumulation as activated macrophages failed to emigrate to local draining lymph nodes through lymphatics.²⁶ This reduced emigration does not seem to take place in skin injury, however, suggesting that Mac-1 is either not critical to macrophage influx or efflux in wounds, or that another molecule compensates. Interestingly, we detected a short term but significant increase in the number of neutrophils in wounds of Mac-1 $-/-$ mice at 12 hours after injury. These findings parallel what has been observed in a corneal wounds²⁷ and peritoneal inflammation²⁸ in Mac-1 $-/-$ mice; this increase in neutrophil content has been suggested to derive from a decrease in neutrophil apoptosis.^{27, 28}

Our finding that small excisional skin wounds of Mac-1 $-/-$ mice heal very normally differs from a previous report that described delayed wound closure in partial-thickness ear wounds and full-thickness skin head wounds of Mac-1 $-/-$ (CD11b null) mice.¹⁷ The differences between these two sets of results indicate that in the face of a genetic perturbation (such as a gene deletion), the wound model, including size and location, impacts healing outcomes. More significantly, the results suggest that small excisional wounds and incisional wounds, as used here, may be more resilient in overcoming genetic deficits. The potential for differing wound models to exhibit dissimilar outcomes in genetically deficient mice has been demonstrated at least once before, as

previous studies showed differential delays in healing between different wound models in TGF- α deficient mice.²⁹⁻³¹ In TGF- α deficient mice, wounds produced by either tail amputation or on the head were found to close normally, while ear wounds exhibited a delay in reepithelialization.

Beyond the wound model, another difference between the current and prior studies in Mac-1 $-/-$ mice is the strain background of the Mac-1 $-/-$ mice. Here we used a commercially available CD11b $-/-$ congenic C57BL/6 strain, while in the prior work the CD11b $-/-$ phenotype was on a mixed strain background. Strain background is infrequently considered as an important variable, despite published evidence for strain variation in healing rates and outcomes.³² In addition to the current and prior study of wound healing in CD11b $-/-$ mice, another previous study has described a wound healing deficit in a Mac-1 deficient mouse strain generated via CD18 deletion.¹⁶ In the CD18 $-/-$ strain of Mac-1 deficiency, wound healing was severely delayed. Macrophage recruitment was not affected, but neutrophils were completely absent in wounds. In addition, TGF- β 1, TGF- β receptor type-II, α -SMA (myofibroblast), and ED-A fibronectin were substantially reduced in the wounds of CD18 null mice. However, this particular CD18 homozygous null mouse strain spontaneously developed severe chronic dermatitis with very few neutrophils. These mice also had elevated circulating neutrophil counts, increased immunoglobulin levels, a severe defect in T cell proliferation, lymphadenopathy, splenomegaly, and abundant plasma cells in skin, lymph nodes, gut, and kidney.³³ The obvious changes in the function of multiple organs and within the immune system suggests that caution is needed in drawing conclusions regarding the impact of Mac-1 deficiency on wound healing using the CD18 $-/-$ mouse model. A comparison of the three wound healing studies (Table 2) in Mac-1 deficient mice suggests that the results in genetically deficient mice can be highly variable and

may be dependent upon wound model, genetic background, and other physiologic abnormalities of the strain.

In the current study, the wounds of Mac-1 deficient mice did exhibit some changes from WT patterns. Mac-1^{-/-} wounds showed significantly downregulated TGF- β 1 production, decreased SMAD2 and SMAD3 expression, decreased granulation tissue, minor decreases in collagen synthesis, and alterations of macrophage phenotype markers. Importantly, though, in assessments of functional healing, small 3 mm diameter excisional and incisional wounds healed nearly normally in Mac-1^{-/-} mice, with normal wound closure, and normal collagen content and architecture. The small excisional skin wounds utilized here do heal with substantial contraction, although reepithelialization does contribute to wound closure in this model.³⁴ Considered in context of prior studies, our results suggest that wounds that heal with contraction, such as the 3mm excisional wounds, and wounds that heal by primary intention, such as incisional wounds, may more easily overcome genetic or other wound healing deficits. These results point to the idea that the relative importance of any single factor in healing wounds may be quite distinct in different types of injury. The concept that only certain types of wounds might manifest delayed healing in the face of genetic deficiency is something that is rarely discussed in studies of genetically deficient strains. Approximately 400 different genetically deficient (often-called knockout) mouse strains have now been examined for wound healing phenotypes. Our data suggests that the conclusions reached from observations in knockout mice should be limited to the specific wound models that were examined, and should not be assumed to be a global observation that is applicable to all wounds. In particular, the absence of any meaningful wound

healing deficiency in a single model may provide incomplete information and should be cautiously interpreted.

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Disclosure Statement: The authors have nothing to disclose.

Table 2. Wound healing in Mac-1 deficient mice (CD11b or CD18 null)

	CD11b -/- Chen, et al, current study	CD11b -/- Sisco, et al, 2007	CD18 -/- Peters, et al, 2005
Viability	Viable, fertile, normal in size,	Viable, fertile, normal in size,	Neonatal death of 10–40%
Immunologic abnormalities	Reduced mast cells in skin; deficient neutrophil function; decreased response to LPS	Reduced mast cells in skin; deficient neutrophil function; decreased response to LPS	Chronic dermatitis; elevated circulating neutrophils and immunoglobulin; T lymphocyte and lymphoid organ abnormalities
Mouse strain	C57BL/6	129SvxC57BL/6	129/SvxC57BL/6
Source of mice	Jackson Laboratory	Coxon, et al, 1996	In house
Sex of mice	Female	?	?
Wound model	3 mm full-thickness dorsal skin wounds	3 mm partial-thickness ear wounds 6 mm full- thickness head wounds	5 mm full-thickness dorsal skin wounds
Wound closure	↔	↓	↓
Re-epithelialization	↔	↓	
Inflammation			
Neutrophils	↑ at 12h		Absent
Macrophages	↔	↔	↔
IL-1 β	↔		
IL-6	↓ at day 1		
TNF- α	↔		
TGF- β 1	↓ at day 7		↓ at day 5

CXCL-1 (KC)	↔		
CCL-2 (MCP-1)	↓ at day 1		
Granulation	↓	↓	
Collagen	↓		
α-SMA	↔		↓
References	26, 27, ³⁵ and ³⁶	26, 27, 35, and 36	16 and 33

No change ↔, decreased ↓, increased ↑. Blank cells indicate that this parameter was not measured.

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Figure legends

Figure 1. Wound closure in Mac-1 deficient and WT mice. A) CD11b expression on neutrophils in day 1 wounds of WT and Mac-1^{-/-} mice. B) CD11b expression on macrophages in day 1 wounds of WT and Mac-1^{-/-} mice. Bar=20 μ m C) Representative photomicrographs of wounds from days 0 to 8 post-wounding. Six 3mm full thickness excisional wounds were made on the dorsal skin of WT C57BL/6 and Mac-1^{-/-} mice. Bar=3mm. D) Representative photomicrographs of HE stained histologic sections of day 1, 3 and 7 wounds. Bar=200 μ m. Arrows indicate wound edges. E) Wound closure expressed as percent of closure as calculated from photomicrographs of wounds. N=10 at each time point and in each group. F) Wound re-epithelialization at days 1 and 3 using histological analysis. N=6 at each time point and in each group except that n=5 in WT group at day 3. G). Volume of granulation tissue at days 5 and 7. N=6 at each time point and in each group except that n=5 in Mac-1^{-/-} group at day 5. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 2. Macrophage content, CCL2 levels, and expression of macrophage phenotype markers in wounds of Mac-1 deficient and WT mice. A) Time course of macrophage density in wounds. B Protein levels of CCL-2 determined by multiplex ELISA, expressed as pg per mg of homogenized tissue. C) mRNA expression of the anti-inflammatory macrophage marker YM1 during the course of wound healing. D) mRNA expression of the pro-inflammatory macrophage marker, iNOS during the course of wound healing. NS: normal or unwounded skin, N=6 at each time point and in each group.

Figure 3. Neutrophil content and levels of CXCL-1, IL-1 β , IL-6, and TNF- α in wounds of Mac-1 deficient and WT mice. A) Time course of the number of neutrophils in wounds. B, C, D, and E) Protein levels of CXCL-1, IL-1 β , IL-6, and TNF- α determined by multiplex ELISA, expressed as pg per mg of homogenized tissue. NS: normal or unwounded skin. N=6 at each time point and group.

Figure 4. Biochemical and mRNA analysis of wound collagen content in Mac1 deficient and WT mice. A) Levels of hydroxyproline in wounds over time. Concentrations of hydroxyproline were determined using a hydroxyproline assay kit. B, C) mRNA expression of collagen I and collagen III over the time course of the healing. NS: normal or unwounded skin. N=6 at each time point and in each group.

Figure 5. Histologic assessment of collagen content in wounds of Mac-1 deficient and WT mice. A) Representative photomicrographs of Masson's Trichrome stained sections of the wound bed at days 7, 14, 21, and 28. Bar=200 μ m. B) Quantification of Masson's Trichrome collagen staining in wounds. The density of blue-stained collagen in wound bed was quantified using Image J. C) Representative photomicrographs of picrosirius red staining sections of normal skin (NS), days 7 and 28 wounds. Bar=100 μ m. D) Summary of quantification of picrosirius red staining. The relative density of collagen I (red-orange) and collagen III (green) in wound bed was quantified using Image J. NS: normal or unwounded skin. N=5 at each time point and in each group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 6. Myofibroblast content and wound breaking strength in wounds of Mac-1 deficient and WT mice. A) Myofibroblast content in day 7 wound bed, assessed as the % of wound area. With α -SMA+ staining. B) Wound breaking strength, in lbs, over the time course of healing. NS: normal or unwounded skin. N=6 at each time point and in each group.

Figure 7. Amount of activated TGF- β 1 protein and mRNA expression of SMAD2&3 in wounds of Mac-1 deficient and WT mice. A) Protein concentrations of activated TGF- β 1 in day 7 wounds as determined by ELISA. B) mRNA expression of SMAD2 and SMAD3 in day 7 wounds as determined by real time PCR. N=6 at each time point and in each group.

Fig. 1

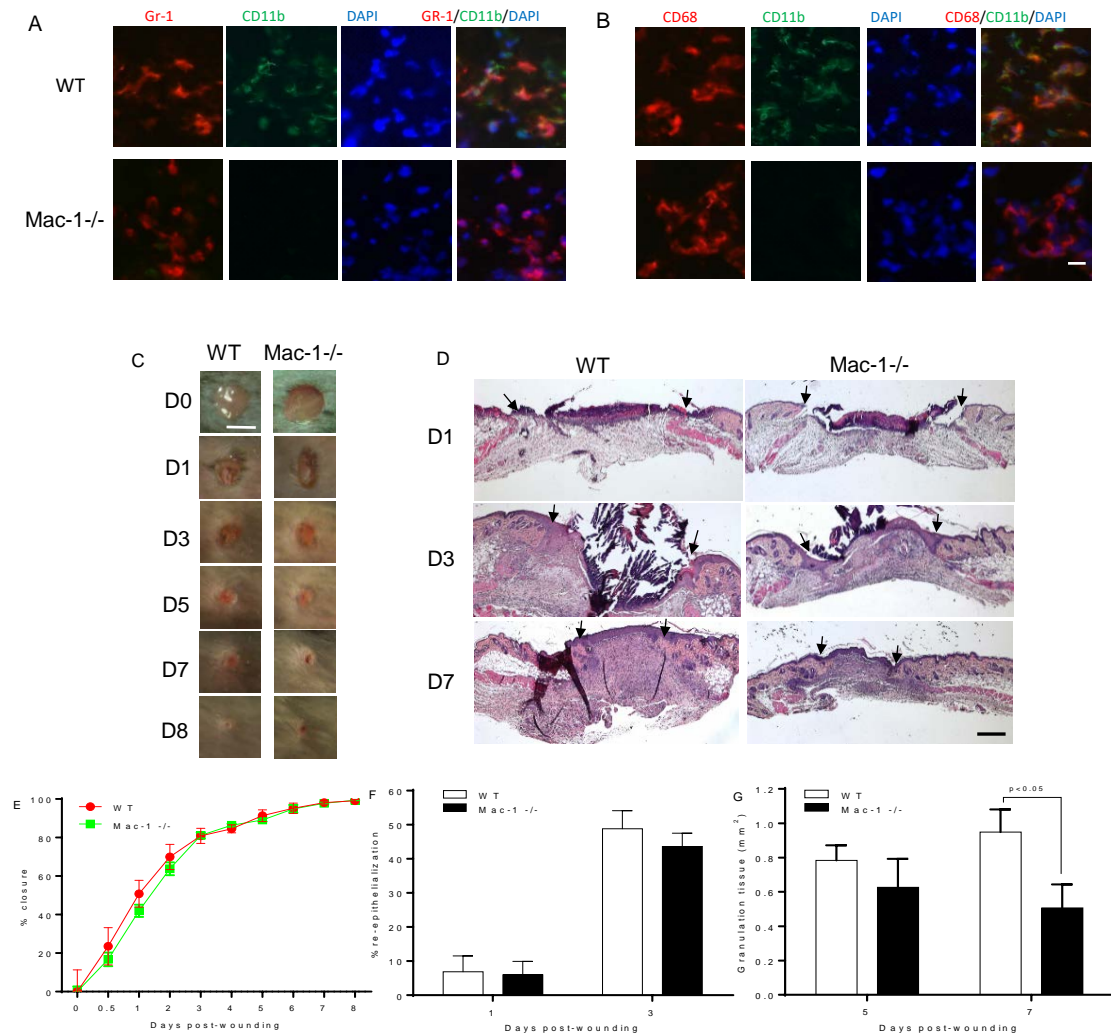


Fig. 2

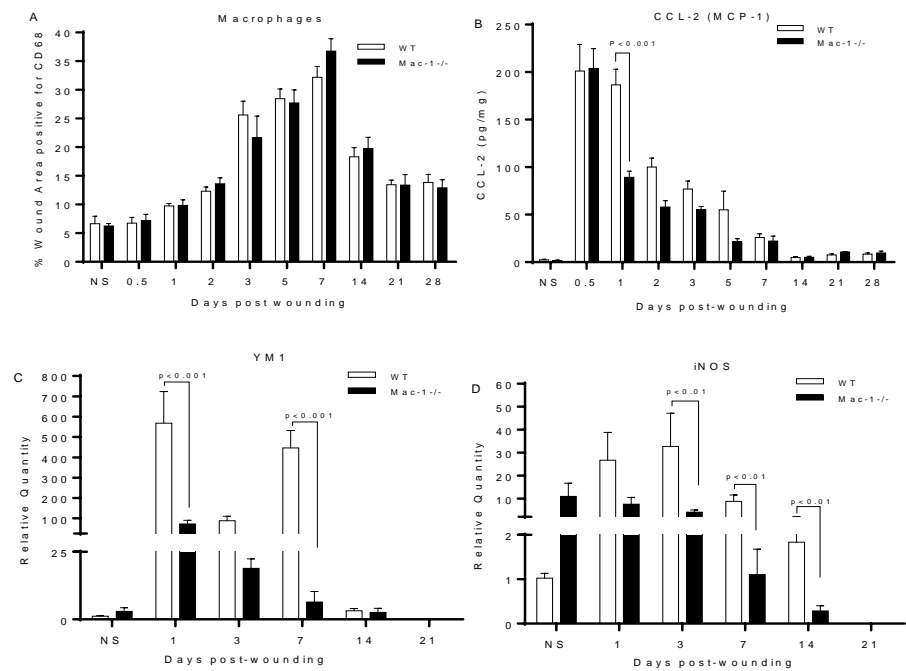


Fig. 3

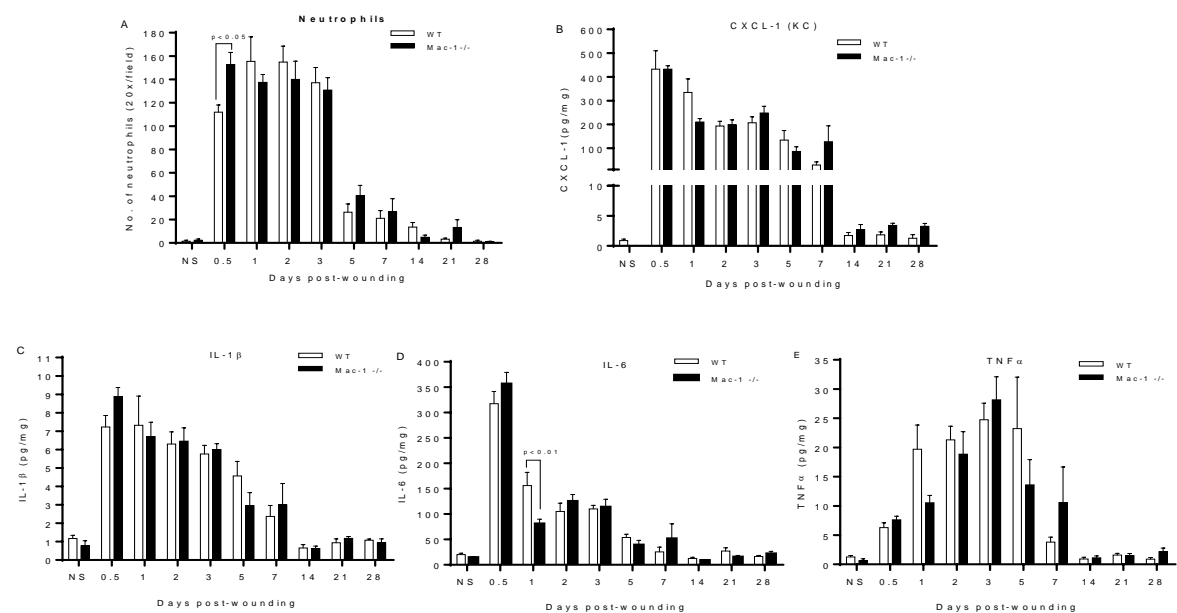


Fig. 4

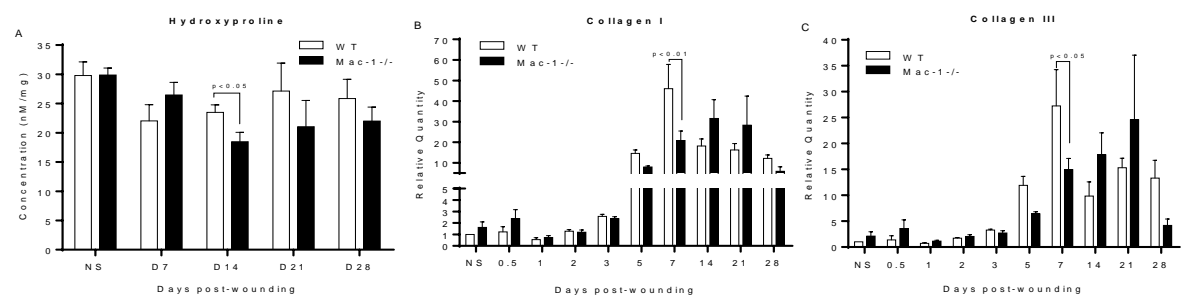


Fig. 5

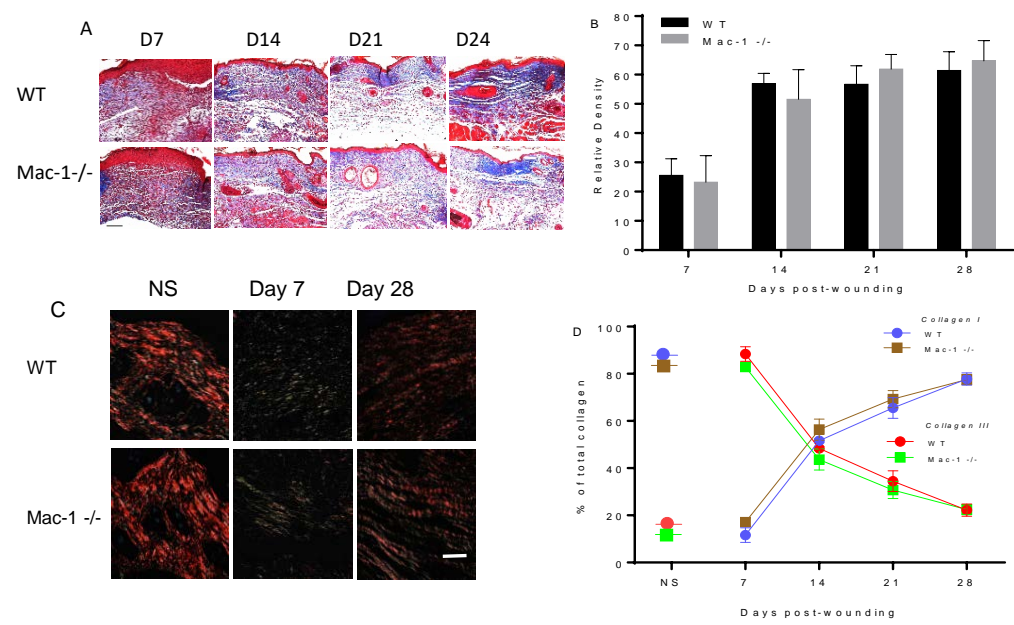


Fig. 6

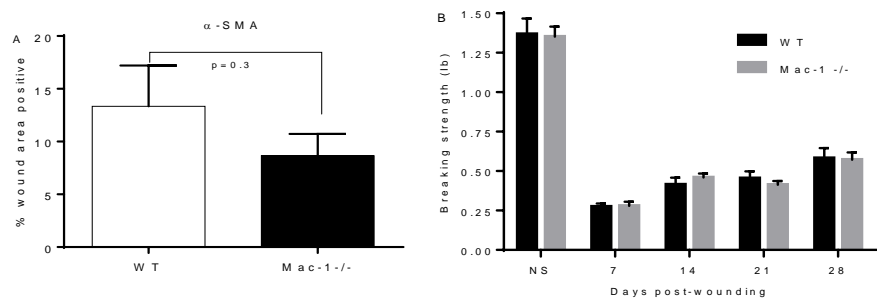


Fig. 7

